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14. ABSTRACT. In this report, we have described the breeding protocol we have followed aimed at knocking out the fetuin-A gene in $PymT^{+}$ transgenic black C57 mice to define the role of fetuin-A in breast cancer carcinogenesis and progression. The central hypothesis of this project is that **fetuin-A is a major serum derived growth factor for breast carcinoma cells and creates a favorable environment for the metastatic spread of tumor cells to the lungs .** .So far we have managed to get $PymT^{+}/Fet^{-/+}$ heterozygous animals that are significantly protected from breast cancer. We therefore hope that the absence of both alleles in $PymT^{+}/Fet^{-/-}$ mice will result in almost total protection of the animals from developing full blown breast cancer. The results are very encouraging and we hope to complete the critical studies by the end of this year. The main problem we have had is the slow pace of the breeding protocol in that most of the $PymT^{+}/Fet^{+/+}$ mothers cannibalize their pups and so it takes many attempts before we can move animals to experimental groups.

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Introduction:

The goal for task1 (Aim # 1) was to define the significance of fetuin-A (ahsg) in breast cancer carcinogenesis. The breeding experiments commenced in the month of July last year after we received the official approval to commence work on June 1, 2007. We are currently crossing $PymT^{+}$ transgenic mice with fetuin-A null animals to generate enough animals which are $PymT^{+}/Fet^{-/-}$; $PymT^{+}/Fet^{+/-}$ and $PymT^{+}/Fet^{+/+}$ to be placed in experimental groups. So far we have had a number of $PymT^{+}/Fet^{+/+}$ female animals live beyond 90 days and all have developed metastatic breast cancer. We have a few female $PymT^{+}/Fet^{-/+}$ animals that have caught our attention. Even though they are approximately 120 days old and still actively breeding, their $PymT^{+}/Fet^{+/+}$ counterparts of the same age have all succumbed to breast cancer. We are following these $PymT^{+}/Fet^{-/+}$ with a lot of interest and will repeat the experiments where we will sacrifice some of the animals at 30, 60, 90 and 120 days to follow the development of mammary hyperplasia if any in these animals. Unfortunately the only $PymT^{+}/Fet^{-/-}$ animals that we have generated so far have been males that are unlikely to develop breast cancer. One drawback in our breeding has been the constant carnibalization of the pups by $PymT^{+}/Fet^{+/+}$ mothers. Currently we have another litter that will be weaned in the next 10 days and of these animals; we hope to have at least one female which will be $PymT^{+}/Fet^{-/-}$.

Based on what we have observed so far with female $PymT^{+}/Fet^{-/+}$ we anticipate that the female $PymT^{+}/Fet^{-/-}$ mice will even live longer and free of cancer. And even if they eventually get breast tumors, the lesions are expected to be smaller and may not metastasize to the lungs.

Task 1- To elucidate the role of fetuin-A in mammary tumorigenesis as assessed by fetuin-A knockout and polyoma middle T antigen transgenic mice (months 1-18).

The central hypothesis of the project is that fetuin-A is a mediator of growth in transformed breast carcinoma cells. Aim # 1 of the study is to define the role of fetuin-A in breast cancer carcinogenesis as assessed by $PymT^{+}/Fet^{-/-}$ transgenic mice. In this task the goal is to cross $PymT^{+}$ transgenic mice with $Fet^{-/-}$ C57/BL-6 with the ultimate goal of assigning at least 12 animals in the control groups ($PymT^{+}/Fet^{+/+}$); $PymT^{-}/Fet^{+/+}$ and 12 animals/group in the experimental groups; $PymT^{+}/Fet^{-/-}$ and $PymT^{+}/Fet^{-/+}$.

We already had some fetuin-A null C57/BL-6 when we obtained funding and so our next step was to obtain $PymT^{+}$ transgenic mice that were also in the C57/BL-6 background. Since most of the transgenic $PymT^{+}$ mice are in FVB/N background, it took us approximately 3 months to finally locate $PymT^{+}$ mice which were in C57/BL-6 background. In fact we contemplated generating our own $Fet^{-/-}$ mice in FVB/N background but then dropped the idea since it would have taken almost 8 months to complete this. We therefore purchased 4 male $PymT^{+}$ mice from Mayo Clinic (Scotsdale campus) in July of last year. Once the animals arrived, they were immediately paired with wild-type C57/BL-6 female mice to generate more $PymT^{+}$ animals. The original female $PymT^{+}$ mice from this generation have since succumbed to breast cancer. The $PymT^{+}$ females are constantly monitored for breast tumor growth from 80 days onwards. The tumors from these animals generally grow rapidly once they attain the age of 90 days and above (Fig. 1). We generally do not allow the tumors to grow beyond 1.5 cm in length (~ mm3) as per the written protocol.

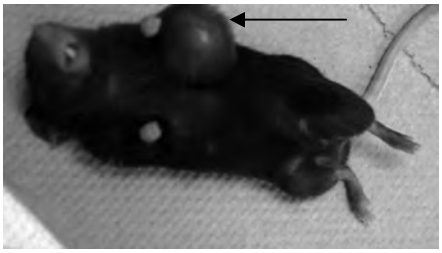

Fig. 1A. *$PymT^{+}/Fet^{+/+}$ female mouse. This is a 100 day old mouse with a large mammary tumor on the left side (arrow) measuring 1.5 cm. in length.*

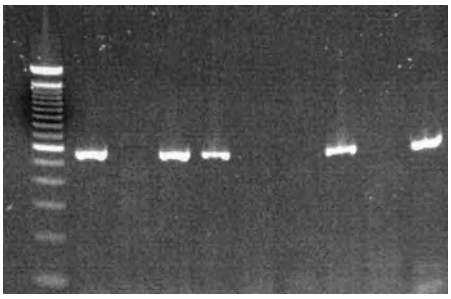

Fig. 1B *is an example of a typical genotyping procedure which in this case identified 5 $PymT^{+}$ (550bp) mice out of a litter of 9.*

Once we obtained enough $PymT^{+}$ males, we crossed them with the female $Fet^{-/-}$ that were kindly donated to us by Professor Johannen Dechent from Aachen, Germany, to obtain the first filial generation (F1) (Fig. 2). All the animals obtained from this cross

were $Fet^{-/+}$. We then crossed the $PymT^{+}$ /$Fet^{-/+}$ males with female $PymT^{+}/Fet^{-/+}$ to obtain the second filial generation (F2). So far in the F2 generation the desired genotype ($PymT^{+}/Fet^{-/-}$) of the mice that we obtained were males. We anticipate to have at least one female $PymT^{+}/Fet^{-/-}$ mice in the next litter to be genotyped. Interestingly we have compared the original $PymT^{+}/Fet^{-/+}$ females that were at least 90 days old to their litter mate controls females that were $PymT^{+}/Fet^{+/+}$. We determined that all the $PymT^{+}/Fet^{-/+}$ females except one are still alive and tumor free at almost 120 days of age. On the other hand all the $PymT^{+/}Fet^{+/+}$ females except one were sacrificed due to tumor burden beginning around day 95 after birth (Table 1). Because of this dramatic turn of events, we expect that when we finally get $PymT^{+}/Fet^{-/-}$ females, they will survive even longer than the $PymT^{+}/Fet^{-/+}$ females. The 4 female $PymT^{+}/Fet^{-/+}$ mice are still breeding and are able to suckle their young ones with no evidence of tumor after 120 days. The only female $PymT^{+}/Fet^{-/+}$ that has tumor is still alive and the tumor is small.

Table 1.

Age	**Genotype**	mice with tumor/tumor free mice
90-120 days old	$PymT^{+}/Fet^{+/+}$	10/11
90-12- days old	$PymT^{+}/Fet^{-/+}$	1/5

The concentration of fetuin-A in the $PymT^{+}/Fet^{-/+}$ serum is approximately half of that in the wild-type animals. Due to our late start in the breeding protocol, we now anticipate that **Task 1a** (start a breeding protocol by crossing $Fet^{-/-}$ C57/BL-6 mice with $PymT^{+}/Fet^{+/+}$ C57/BL-6 animals), will take 1-18 months instead of the original 1-5 months.

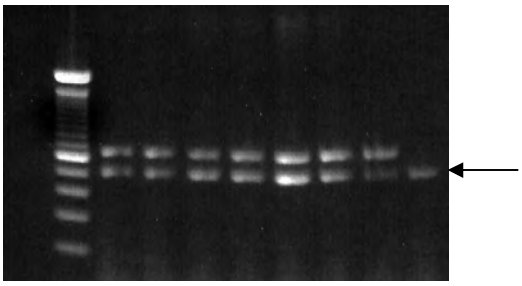

Fig. 2. *<u>The genotype of the first filial generation with respect to fetuin-A</u>. All the mice are heterozygous with the lower band of 550 bp representing the wild type and the upper band 650 bp(neo) representing null. The last lane (lane 8) represents the wild-type control. Lanes 3, 4, 5, and 7 were also $PymT^{+}$. These were then crossed (brother and sister) to generate the second filial generation.*

We have completed the histopathology of the breast tumors obtained from the $PymT^{+}/Fet^{+/+}$ mice. At day 30, we can already observe mammary hyperplasia lesions, characterized by increase in the number of epithelial layers lining the mammary glands (arrow) as depicted in Fig.3A. At day 95, mammary intraepithelial neoplasia (MIN), characterized by acini consisting of tightly packed layers of neoplastic cells surrounded by fibrous connective tissue (arrow), is evident (Fig. 3B). Note that MIN is surrounded by adipose tissue and granulocytes. Mice that have tumors (Day 95) also show evidence of metastatic spread of the tumors to the lungs. Secondary lung nodule, characterized by

epithelial ductal lesion in the alveoli space (arrow) surrounded by high density leukocytic infiltration throughout can be observed in Fig. 3C. In other mice where the breast tumors have grown to a large size ~ 1.5 c.m. in length, extensive colonization of the lung by the tumor cells is quite obvious. Solid secondary adenocarcinoma lung nodule, characterized by tightly packed sheets of cells composed of pleomorphic populations of neoplastic cells with numerous mitotic cells can be seen in Fig. 3D.

In between breeding and genotyping protocols, we have also began studies pertaining to Task 2a, where we analyze the role of serum fetuin-A (ahsg) in the growth of tumor cells. We have demonstrated that whereas serum proteins from breast cancer patients can support the adhesion and growth of tumor cells in monolayer cultures, fetuin-A from this serum in particular appears to support mainly anchorage independent growth. If tumor cells are allowed to grow in serum free medium in the presence of fetuin-A purified from the serum, the cells tend to grow in clumps. This type of growth is also seen if tumor cells are exposed to vesicular structures from human serum and urine that are also loaded with fetuin-A (Fig. 4).

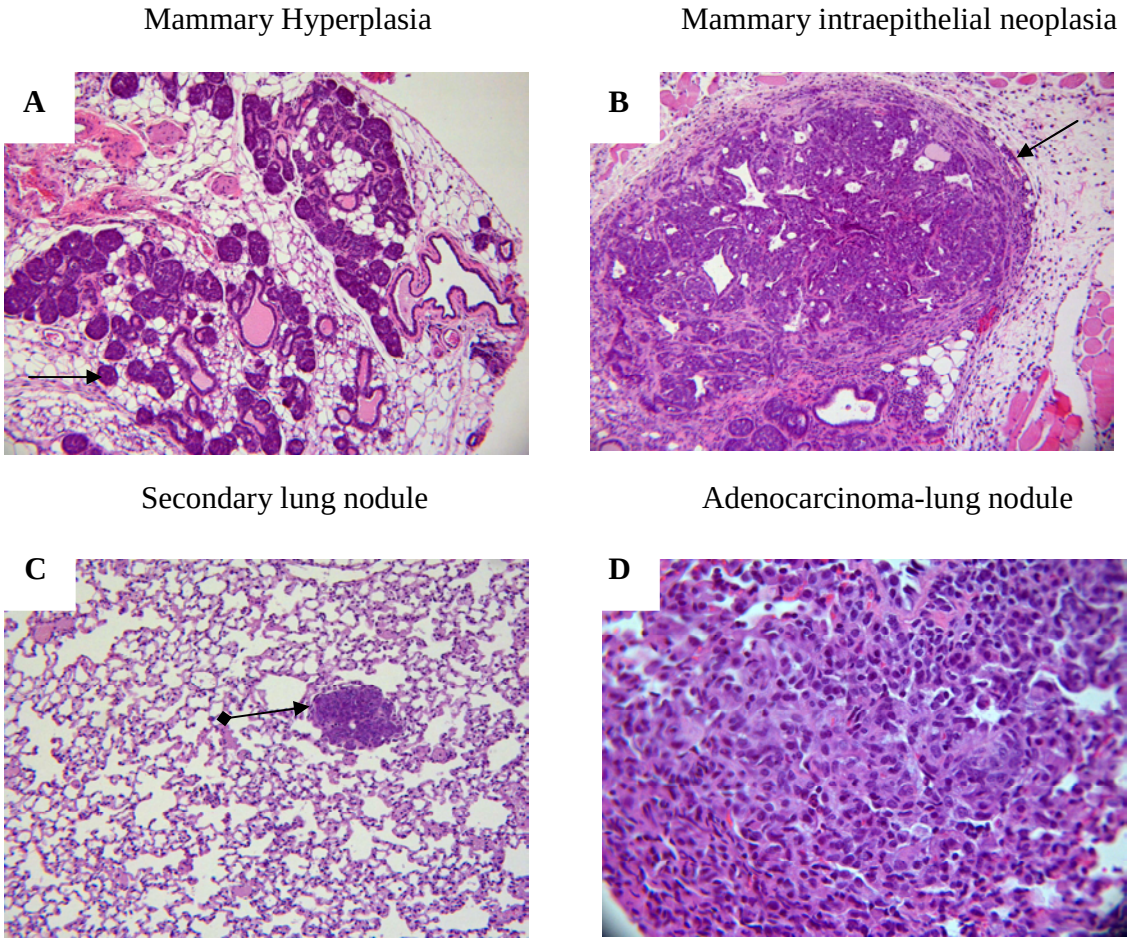

Fig. 3. *Histopathology of mammary tumors in PymT^{+}/Fet$^{+/+}$ animals.*

The vesicular structures also known as exosomes are of great interest to us since they act as growth platforms for breast tumor cells. We have mapped the proteins in them by mass spectrometer (services provided by our proteomics core facility and Vanderbilt Proteomics laboratory) and determined that apart from fetuin-A, they also contain mainly immunoglobins and other acute phase proteins. If antibodies to fetuin-A (ahsg) are added to the cells growing either in the presence of purified fetuin-A or serum exosomes, growth is significantly compromised indicating that fetuin-A either in solution or associated with these vesicular structures is somehow responsible for the anchorage independent growth. Our current experiments are designed to determine how fetuin-A by itself or anchored on exosomes can mediate the anchorage independent growth of breast tumor cells.

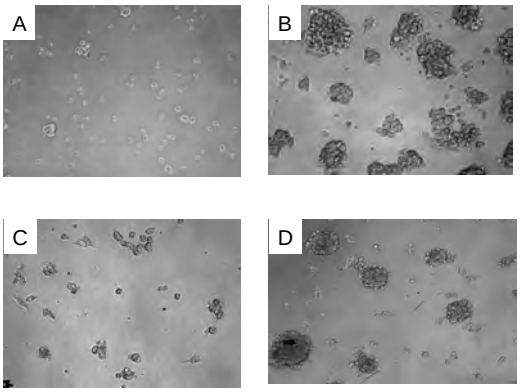

Fig. 4. *Growth of tumor cells in the presence of serum and urinary exosomes. Breast tumor cells (BT-549-gal3) were cultured in 96-well microplate at 1,000 cells/well in serum free medium and allowed to grow for at least two weeks in the absence (panels A and C) or in the presence of serum (panel B) or urine (panel D) derived exosomes.*

Key Accomplishments:

- The fact that mice that are $PymT^{+}/Fet^{-/+}$ are protected from breast cancer is highly significant and supports the central hypothesis. We therefore anticipate that mice that are $PymT^{+}/Fet^{-/+}$ will even be more protected relative to the $PymT^{+}/Fet^{+/+}$ controls.

- The ability of exosomes in serum that are decorated with fetuin-A to support the anchorage independent growth of tumors is the other key finding of this project and is beginning to give us a hint as to which growth signaling pathway/s fetuin-A is involved in.

Pitfalls:

One of the major pitfalls that we did not anticipate when we started this project is the difficulty in breeding $PymT^{+}$ transgenic mice. Most of the female that are $PymT^{+}/Fet^{+/+}$ tend to cannibalize their young ones immediately after birth. We have tried all sorts of combinations but cannot seem to get around this problem. This is why we have not been able move $PymT^{+}/Fet^{-/-}$ animals from breeding to experimental protocols. However, we are having success with $PymT^{+}/Fet^{-/+}$ females who are cancer free even after 120 days. It will take us longer than we had anticipated completing the experimental protocols.

Reportable Outcomes:

We anticipate to submit our first manuscript pertaining to the animal experimentation after we complete the first set of experiments, which will include comparing tumor incidence in $PymT^{+}/Fet^{+/+}$; $PymT^{+}/Fet^{-/+}$; and $PymT^{+}/Fet^{-/-}$ experimental groups. Based on what we have observed so far, the data support our central hypothesis and so we will carefully analyze all the data to make sure the results we are reporting represent solid data that will withstand the test of time. Regarding the *in vitro* studies, we hope to submit the manuscript in the next two months.

Conclusion:

We are indeed gratified with the direction of research. Even though we started the animal experimentation (Task 1) later than we had planned, the progress has been very rapid and we sincerely hope to complete Task 1A towards the end of the year.